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13. ABSTRACT ***(Maximum 200 Words)***

BRCA2 hereditary gene mutations confer a profound predisposition to breast cancer in women. We have generated mice carrying a *Cre-loxP* conditional *Brca2* mutated allele by flanking exon 27 with *loxP* sites and we predict that this will disrupt basic functions of *Brca2* in DNA repair. The mammary-specific removal of *Brca2* exon 27 by *Cre*-mediated recombination *in vivo* was performed by crossing the homozygous floxed *Brca2* mice with MMTV-*Cre* transgenic mice. The effect of this mutation on the formation of mammary gland tumors and alterations in mammary gland morphogenesis is currently being examined for both untreated and radiation-treated animals. Mice with a homozygous germline deletion of *Brca2* exon 27 have also been created by transiently electroporating embryonic stem cells carrying the floxed *Brca2* allele with a *Cre*-expression plasmid. These studies have generated the first completely viable germline homozygous *Brca2* knockout mice. Female mice with this homozygous *Brca2* disruption display an inhibition of ductal morphogenesis in the mammary gland by six months of age, which may be an early biological marker for cancer susceptibility. Parallel studies using these two distinct animal models for *Brca2* inactivation should provide valuable insights into the functional role of *Brca2* in the breast and throughout the body.

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Table of Contents

Mammary-Specific Targeting of the *Brca2* Breast Cancer Susceptibility Gene in Mice

Introduction:

The breast cancer susceptibility gene BRCA2 is known to be responsible for a substantial portion of inherited breast cancer. An appropriate animal model is necessary to determine how specific defects in *Brca2* strongly predispose to breast tumorigenesis. The early embryonic lethality of *Brca2* null mice that we had previously generated impeded functional analyses of *Brca2* in normal mammary gland development and its role in neoplasia. We therefore proposed to generate mice carrying a conditional *Brca2* mutation whereby *Brca2* would be disrupted specifically in the mammary tissue by gene targeting with the *Cre-lox*P system. We hope these mice with a mammary-specific mutation in *Brca2* will closely mimic women that have inherited one defective breast cancer gene and develop a secondary mutation later in life in the breast. We are currently assessing these mice to define the phenotypes associated with the loss of *Brca2* function during normal and neoplastic development of the mammary gland and specifically, to clarify the effects of radiation on breast cancer risk in a properly controlled environment without the genetic variation intrinsic to the human population. We have also generated the first completely viable homozygous *Brca2* germline knockout mice. These mice display an obvious inhibited mammary ductal branching morphology by six months of age which may be an early biological marker for breast cancer susceptibility. These two distinct animal models for breast tumorigenesis, as well as various cell lines originating from them, are being used to further identify and clarify the role of *Brca2* in DNA damage repair pathways and its role in mammary tumorigenesis.

Body:

Statement of Work Summary: We have now completed Tasks 1, 2, 3 of Technical Objective 1: Development of mammary gland-specific *Brca2*-deficient mice. Mice with a mammary-specific mutation in exon 27 of *Brca2* have now been generated as well as a homozygous germline Δ27 *Brca2* mutation. We are currently completing Task 4 of Technical Objective 1 by establishing both the *Brca2* (flox) and germline Δ27 *Brca2* mouse lines on various inbred strain backgrounds including 129Sv/Ev, C57Bl/6J, Balb/cJ, and SWR/J, which have different susceptibilities to mammary carcinogenesis. We have currently begun to utilize the technique of "speed congenics" to create congenic strains of this conditional and germline knockout in a reduced amount of time and with more accuracy than through traditional breeding protocols (Markel, 1997; Wakeland, 1997).

We have begun the long-term analysis of the results of both this mammary-specific and germline *Brca2* mutation on mammary gland tumor formation and mammary gland morphogenesis as stated in Task 1 of Technical Objective 2: Analyses of mammary gland-specific *Brca2*-deficient mice. We have also begun to isolate mammary epithelial cells from these animals for the proposed *in vitro* work proposed in Task 2 and 3 of Technical Objective 2. We have isolated murine embryonic fibroblast cultures from wildtype and homozygously deleted exon 27 *Brca2* cells and are presently beginning to

use various cDNA microarray technologies as well as quantitative RT-PCR analysis to compare gene expression levels between these distinct population of cells (as stated in Task 4, Technical Objective 2). Finally, we have begun several long-term studies attempting to assess the impact of radiation exposure for our conditional and germline *Brca2* knockout animals (as stated in Task 5, Technical Objective 2). Serial sacrifices are presently being performed on a three-month basis to monitor potential preneoplastic mammary lesions and other alterations in mammary gland morphology for these two animal models.

Specific Aim 1, 2, and 5:

We have strived to generate an appropriate animal model for breast cancer by disrupting the *Brca2* gene in the mouse. Our original attempt to generate a *Brca2* knockout mouse (by introducing a stop codon into exon 10) resulted in an early (day 7-9) embryonic lethal phenotype in the homozygous state which is modified by genetic strain background (Bennett, et al. 2000-reprint included in appendice). We have now generated a conditional knockout mouse model for *Brca2* that would allow the deletion of the final exon of the gene at a later time in the mammary gland specifically.

In the generation of this conditional *Brca2* mouse knockout, the mouse homologue of BRCA2 has been disrupted through the use of a targeting construct, which has the final exon of the gene, exon 27, flanked by loxP sites (Figure 1). A single loxP site was inserted into intron 26 and into a downstream region beyond the *Brca2* stop codon. This "floxed" targeting vector was introduced into ES cells by electroporation and a properly targeted ES cell clone was identified. Germline transmission of the *Brca2* floxed allele was then obtained using standard knockout protocols and procedures and the homozygous floxed animals were confirmed to have no detrimental phenotype. The puberty-specific deletion of *Brca2* exon 27 in the mammary tissue has been performed by crossing homozygous *Brca2* floxed mice with an MMTV-Cre transgenic mouse strain. The Cre recombinase activity is restricted to mammary tissues by activation of a murine mammary tumor virus (MMTV) promotor with the onset of ovarian function during puberty in this transgenic mouse strain (Wagner, et al. 1997).

A successful colony of these animals that should become defective for *Brca2* function specifically in the mammary gland during puberty have now been generated. We are currently assessing the percentage of mammary epithelial cells that have the disruption of *Brca2* in the adult virgin animals. We have also been able to confirm the activation of Cre in the mammary gland epithelial cells during the onset of puberty for the MMTV-Cre transgenic strain (MMTV-Cre strain D) we have used by taking advantage of a Cre reporter mouse strain (Soriano, 1999) whereby Cre-mediated lacZ expression can be detected by standard histological procedures (Wagner, *et al.*, submitted manuscript enclosed in appendice). This has allowed us to be confident that *Brca2* inactivation in this conditional knockout model would occur predominantly in the target mammary epithelial cells.

The conditional knockout animals and their corresponding age-matched controls are presently being examined for distinct mammary gland changes with and without the additional environmental insult of irradiation. Approximately 60 animals of each genotype were irradiated with 5 Gy at 5 weeks of age. All mammary gland alterations

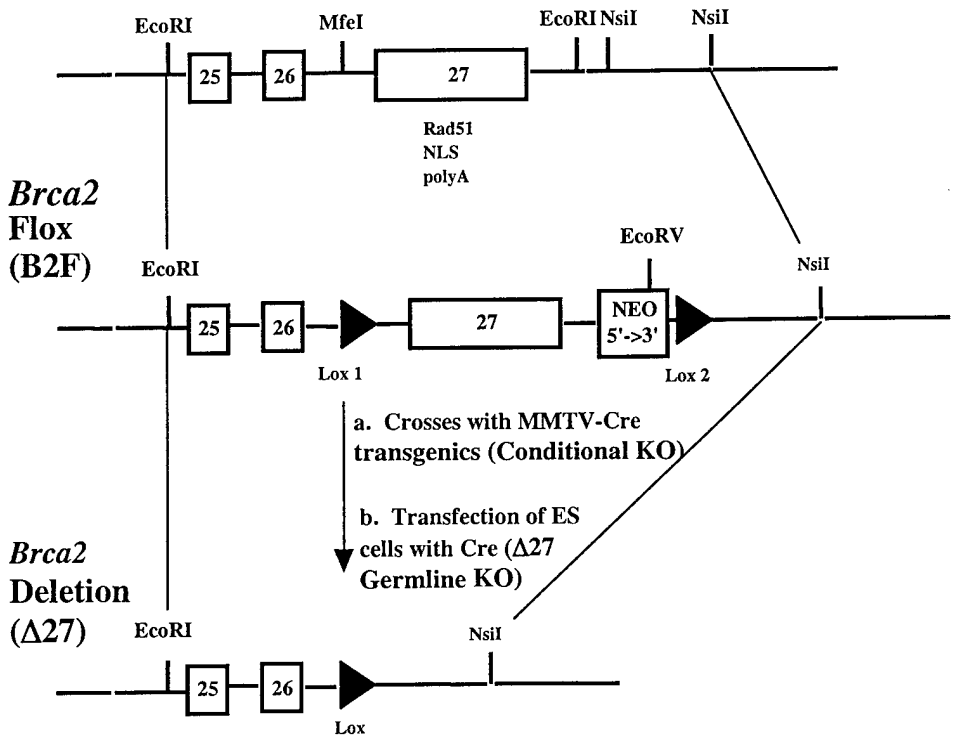

Figure 1. Conditional and Germline *Brca2*$^{\Delta 27}$ Targeting Strategies

The endogenous genomic *Brca2* gene (Wildtype *Brca2* Locus) is compared to the *Brca2* floxed targeted allele (B2F) and the *Brca2* exon 27 deleted allele (Δ27). The addition of the Cre recombinase protein will allow the site-specific recombination to occur and removal of exon 27 as well as the neomycin gene will result. A mammary-specific conditional knockout of exon 27 *Brca2* was generated by crossing animals homozygous for the floxed *Brca2* allele to MMTV-Cre transgenic animals. A germline knockout of exon 27 *Brca2* was generated by initially creating Δ27 ES cells by transient electroporation of the floxed ES cells with a Cre plasmid.

including preneoplastic changes and mammary tumor incidence for these mice will be assessed. Mice from each genotypic class are being sacrificed presently at interim dates (2, 3, 6, 9, 12, and 15 months of age).

A preliminary pilot experiment is also ongoing to attempt to investigate the role of various reproductive events associated with breast cancer risk or protection in humans. In addition to following a hundred virgin *Brca2* conditional animals, the effect of pseudopregnancy and full-term pregnancy with or without lactation is currently being analyzed in these animals as well. Twenty female mice of each informative genotype have been housed with vasectomized males to induce and maintain a pseudopregnant state. Ten female mice of each informative conditional *Brca2* genotype have been bred with males through at least three rounds of pregnancy without lactation, forcing them to undergo multiple cycles of pregnancy induced proliferation and differentiation followed by involution. Finally, twenty female mice of each informative conditional *Brca2* genotype are being continuously bred with males and allowed to lactate until natural weaning of pups. These female animals will not be forced to undergo involution until three cycles of pups have been raised. A final 12-month and 15-month timepoint on these animals is presently being performed.

In parallel studies, I have also generated a germline deletion of *Brca2* exon 27 by transiently transfecting embryonic stem cells carrying the conditional *Brca2* allele with *Cre*. These experiments have created the first known germline homozygous *Brca2* knockout animals that appear to be completely viable up to twelve months of age. Preliminary results from a six month timepoint sacrifice of six animals show an obvious inhibited ductal branching morphology in the mammary glands of three homozygous Brca2 mutant animals compared to one wildtype and two heterozygous littermates (Figure 2). We believe that these results suggest the likelihood of an increased susceptibility to mammary tumor formation after a longer latency period or in combination with additional carcinogenic exposures or environmental insults based on the results from previous *Brca1* animal models. Germline and conditional homozygous *Brca1* animals initially displayed a similar severe inhibition of mammary ductal branching (Gowen, et al. 1998; Cressman, et al. 1999; Xu, et al. 1999). The conditional *Brca1* mice (which were generated with the same MMTV-Cre transgenic mice which we are utilizing for our conditional *Brca2* knockout studies) developed subsequent mammary tumor formation after a long latency period (10-13 months of age) with pathology similar to human breast cancer. We are currently analyzing whether this inhibited branching pattern is less or more pronounced in pregnant homozygous *Brca2* mutant animals compared to the virgin animal controls. We are also currently analyzing various additional phenotypes for these animals including a partial disruption of spermatogenesis and alterations in the prostate gland development with collaborators Dr. Mitch Eddy, NIEHS and Dr. Gail Prins, U. of I., Chicago.

Given the surprising viability of the homozygous Δ27 *Brca2* knockout animals, a similar experimental design to examine the effects of radiation was initiated for these animals as was performed for the conditional Δ27 *Brca2* knockouts. We believe the susceptibility of the *Brca2* mutant animals to mammary gland tumorigenesis will be enhanced by placing the animals on a p53-deficient background. We have therefore initiated a long-term study irradiating five-week old animals at 5 Gy that are either heterozygous or homozygous for one or both the exon 27 deletion of *Brca2* as well as a

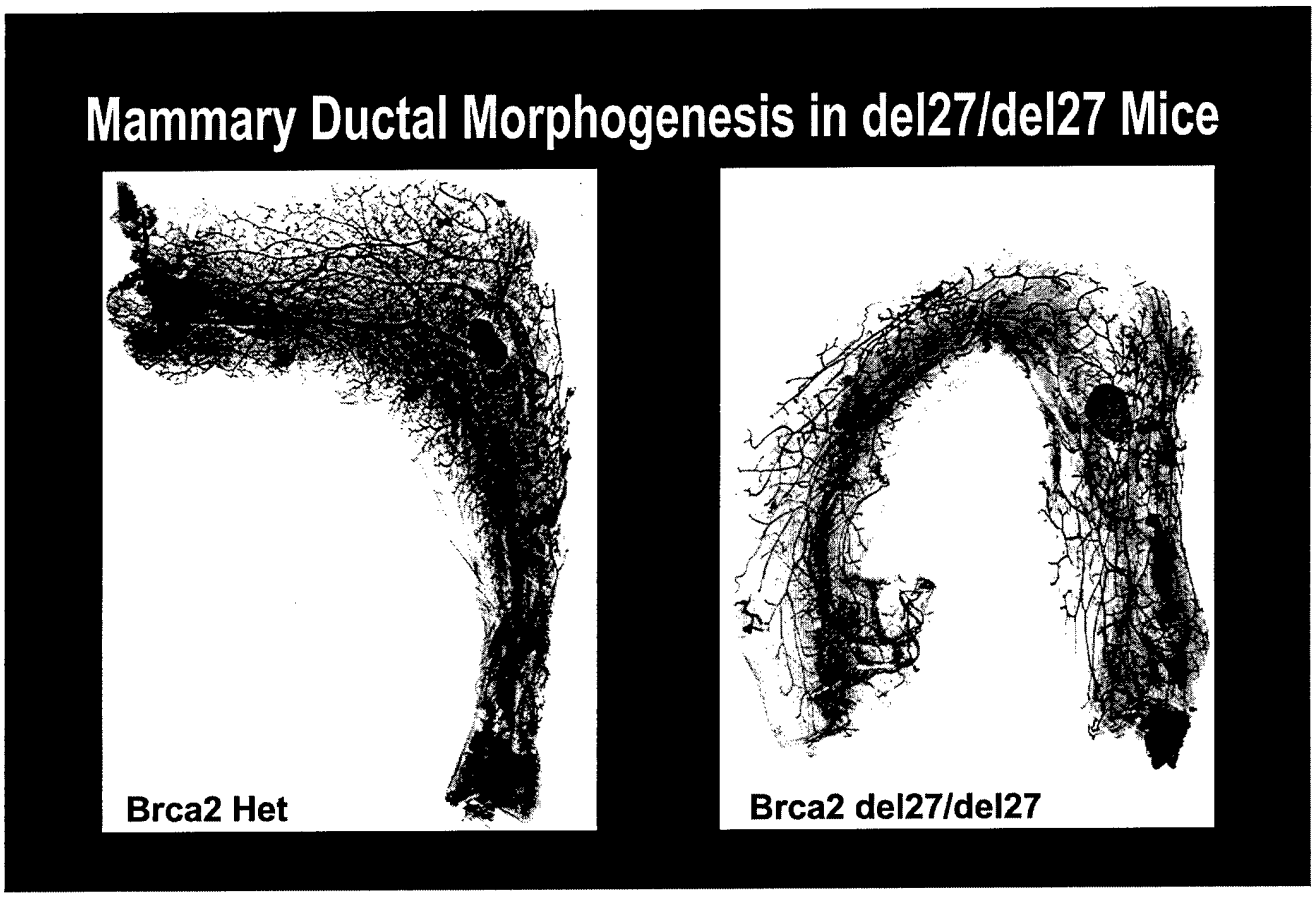

Figure 2. Alterations in Mammary Gland Morphogenesis for *Brca2*$^{\Delta 27/\Delta 27}$ Knockout Animals
An inhibition of branching in a mammary gland whole mount taken from a 6-month old homozygous Δ27*_Brca2* animal is observed compared to a representative heterozygous 6-month littermate. We believe this phenotype may be an early biomarker for mammary tumor susceptibility. This delay in development will very likely result in an increased susceptibility to mammary tumor formation after a longer latency period or in combination with additional carcinogenic exposures or environmental insults based on the results from previous *Brca1* animal models. Germline and conditional homozygous *Brca1* animals initially displayed a severe inhibition of mammary ductal branching (Gowen, *et al.* 1998; Cressman, *et al.* 1999; Xu, *et al.* 1999).

P53 null mutation. Preliminary results suggest a substantial morbidity for the homozygous p53 null animals.

Specific Aim 3 and 4:

Murine embryonic fibroblast (MEF) cultures have recently been generated from intercrosses of heterozygous germline *Brca2* knockout animals as well as intercrosses of double heterozygous *Brca2*/p53 knockout animals and the growth rates and sensitivity to radiation are currently being assessed for these cells. We have also recently initiated collaborations with Dr. Fergus Couch, Mayo Clinic, to assess the apoptosis rates of these cells as well. Preliminary experiments are also ongoing to attempt to establish murine mammary epithelial cells from the conditional and germline exon 27 *Brca2*-deficient animals as these cells are believed to be the key target cell type susceptible to mammary tumorigenesis in humans and mice. I have also generated embryonic stem cells that are homozygous for the deletion of exon 27 *Brca2* by retargeting of the Δ27 ES cells with the flox targeting construct followed by electroporating with a Cre plasmid. I have begun to do initial analyses of these cells growth characteristics and deficiencies in DNA repair as well.

We are interested in examining the specific changes in gene expression for *Brca2*-deficient cells compared to wildtype controls. We plan to utilize the murine cDNA microarray system established by the NIEHS cDNA Microarray Center to examine differences in gene expression patterns for the homozygous Δ27 *Brca2* MEFs (compared to control wildtype MEFs). We have also begun to use the Atlas ™ cDNA expression arrays (Clontech) to generate preliminary expression profiles for the homozygous Δ27 *Brca2* MEFs (compared to controls). We have also begun to develop a quantitative RT-PCR analysis of genes of interest including p53 and p21. We hope to be able to extend these studies using mammary epithelial cells in the future as well. Finally, we would also like to correlate these *in vitro* studies to *in vivo* alterations by directly examining mammary gland tissue from these animals for gene expression analyses in the future.

Key Research Accomplishments:

1) Embryonic lethality of the initial Δ10/11 *Brca2* mutation shown to be altered by genetic background

2) Generation of mice with germline transmission of the floxed *Brca2* allele; Subsequent mammary-specific deletion of *Brca2* generated by crossing *Brca2* floxed mice with MMTV-Cre transgenic mice

3) Generation of germline homozygous Δ27 *Brca2* mice and defined mammary gland and spermatogenesis phenotypes identified

4) Specificity of MMTV-Cre strain D transgene confirmed with the use of LacZ (Rosa26) reporter mice

5) Generation of homozygous Δ27 *Brca2* ES cells and murine embryonic fibroblast cultures for *in vitro* studies

Reportable Outcomes:

Bennett, L.M.*, McAllister, K.A.*, Blackshear, P.E., Malphurs, J., Collins, N.K., Ward, T., Bunch, D.O., Goulding, G., Gowen, L, Koller, B., Eddy, M.E., Davis, B.J., and Wiseman, R.W. *Brca2*-Null Embryonic Survivability is prolonged on the BALB/c Genetic Background. Molecular Carcinogenesis 28: 174-183, 2000 (Reprint included).

Thangaraju, M., Wu, K., Kottke T., McAllister, K.A., Wiseman, R.W., Ingle, J.N., Lingle, W., Kaufmann, S.H., and Couch, F.J. BRCA2 modulates the apoptotic response to cellular stress. Submitted to EMBO, 2000.

Wagner, K.-U.*, McAllister, K.A.*, Ward, T., Davis, B., Wiseman, R., and Hennighausen, L. Spatial and temporal expression of the Cre gene under the control of the MMTV LTR in different transgenic lines. Submitted to Transgenic Research, 2000 (Manuscript included).

Flores, K. G., McAllister, K.A., Greer, P.K., Wiseman, R.W., and Hale, L. P. BRCA2 is differentially expressed during thymocyte development and regulates thymocyte apoptosis. Manuscript in progress, 2000.

Poster presentations of this research occurred in this past year at the 2000 AACR meeting, the Mammary Gland Gordon Conference, and the Department of Defense Era of Hope meeting.

Author applied for a NIEHS TIP (Transition to Independent Position) grant this summer through a career transition (K22) mechanism based on work supported by this award.

Conclusions:

To overcome the original embryonic lethality of an initial exon 10/11 *Brca2* disruption and develop a more appropriate animal model for breast cancer development, mice carrying a *Cre-lox*P conditional *Brca2* mutated allele have now been generated by flanking exon 27 with *lox*P sites. These *Brca2* conditional mice are crossed with mice carrying an MMTV-*Cre* transgene, which allows the disruption of *Brca2* to occur specifically in the mammary gland during the onset of puberty. This animal model should therefore closely mimic women who have inherited one BRCA2 defect but only later acquire a secondary BRCA2 mutation in the breast. In parallel studies, I have also generated a germline deletion of *Brca2* exon 27 by transiently transfecting embryonic

stem cells carrying the conditional *Brca2* allele with *Cre*. These experiments have created the first known germline homozygous *Brca2* knockout animals that appear to be completely viable up to twelve months of age. Preliminary studies suggest, however, that these animals display an inhibition of secondary ductal branching in the mammary gland by six months of age. These conditional and germline *Brca2* knockout animals are both currently being monitored carefully for mammary tumorigenesis and additional alterations in mammary gland development.

Parallel studies using these two distinct animal models for *Brca2* inactivation should provide valuable insights into the functional role of *Brca2* in the breast and throughout the body. The establishment of these two *Brca2* mouse lines on various inbred strain backgrounds has been initiated to examine genetic background effects to mammary carcinogenesis. We believe placing the germline *Brca2* knockout animals on a p53-deficient background may enhance the susceptibility of these animals to mammary tumorigenesis and we have therefore recently initiated these long-term studies. We also believe these conditional and homozygous germline knockout animals may be particularly sensitive to radiation and we have therefore also initiated long-term studies to examine the effects of radiation for these mice. I have isolated homozygous Δ27 *Brca2* embryonic fibroblast cells as well as embryonic stem cells in order to begin to analyze the unique *in vitro* properties of these *Brca2*-deficient cells. I am currently isolating mammary epithelial cells from these animals as well. I hope this distinct targeted population of cells might be particularly useful as a resource to investigate various gene-gene and gene-environment interactions for *Brca2* deficiency using such currently developing technologies as cDNA microarray analysis.

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Bennett, L.M., McAllister, K.A., Blackshear, P.E., Malphurs, J., Collins, N.K., Ward, T., Bunch, D.O., Goulding, G., Gowen, L, Koller, B., Eddy, M.E., Davis, B.J., and Wiseman, R.W. *Brca2*-Null Embryonic Survivability is prolonged on the BALB/c Genetic Background. Molecular Carcinogenesis 28: 174-183, 2000.

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Wagner, K.-U., McAllister, K.A.,_Ward, T., Davis, B., Wiseman, R., and Hennighausen, L. Spatial and temporal expression of the Cre gene under the control of the MMTV LTR in different transgenic lines. Submitted to Transgenic Research, 2000.

Wakeland, E., Morel, L., Achey, K., Yui, M., and Longmate, J. Speed Congenics: a classic technique in the fast lane (relatively speaking). Immunology Today 18: 472-277, 1997.

Xu, X., Wagner, K.-U., Larson, D., Weaver, Z., Li, C., Ried, T., Hennighausen, L., Wynshaw-Boris, A., and Deng, C.-X. Conditional mutant of Brca1 in mammary epithelial cells results in blunted ductal morphogenesis and tumour formation. Nature Genetics 22: 37-42, 1999.

Appendices:

Enclosed:

(1) Bennett, L.M., McAllister, K.A., Blackshear, P.E., Malphurs, J., Collins, N.K.,Ward, T., Bunch, D.O., Goulding, G., Gowen, L, Koller, B., Eddy, M.E., Davis, B.J., and Wiseman, R.W. *Brca2*-Null Embryonic Survivability is prolonged on the BALB/c Genetic Background. Molecular Carcinogenesis 28: 174-183, 2000. (Reprint)

(2) Wagner, K.-U., McAllister, K.A.,_Ward, T., Davis, B., Wiseman, R., and Hennighausen, L. Spatial and temporal expression of the Cre gene under the control of the MMTV LTR in different transgenic lines. Submitted to Transgenic Research, 2000. (Manuscript)

MOLECULAR CARCINOGENESIS 28:174–183 (2000)

Brca2-Null Embryonic Survival is Prolonged on the BALB/c Genetic Background

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Women who inherit mutations in the BRCA2 cancer susceptibility gene have an 85% chance of developing breast cancer. The function of the BRCA2 gene remains elusive, but there is evidence to support its role in transcriptional transactivation, tumor suppression, and the maintenance of genomic integrity. Individuals with identical BRCA2 mutations display a different distribution of cancers, suggesting that there are low-penetrance genes that can modify disease outcome. We hypothesized that genetic background could influence embryonic survival of a Brca2 mutation in mice. Brca2-null embryos with a 129/SvEv genetic background ($129^{B2-/-}$) died before embryonic day 8.5. Transfer of this Brca2 mutation onto the BALB/cJ genetic background ($BALB/c^{B2-/-}$) extended survival to embryonic day 10.5. These results indicate that the BALB/c background harbors genetic modifiers that can prolong Brca2-null embryonic survival. The extended survival of $BALB/c^{B2-/-}$ embryos enabled us to ask whether transcriptional regulation of the Brca1 and Brca2 genes is interdependent. The interdependence of Brca1 and Brca2 was evaluated by studying Brca2 gene expression in $BALB/c^{B1-/-}$ embryos and Brca1 gene expression in $BALB/c^{B2-/-}$ embryos. Nonisotopic in situ hybridization demonstrated that Brca2 transcript levels were comparable in $BALB/c^{B1-/-}$ embryos and wild-type littermates. Likewise, reverse transcriptase–polymerase chain reactions confirmed Brca1 mRNA expression in embryonic day 8.5 $BALB/c^{B2-/-}$ embryos that was comparable to *Brca2*-heterozygous littermates. Thus, the Brca1 and Brca2 transcripts are expressed independently of one another in Brca1- and Brca2-null embryos. *Mol. Carcinog. 28:174–183, 2000.*

Key words: Brca2, BALB/c, gene targeting, embryonic lethality

INTRODUCTION

Genetic inheritance represents a major risk factor for breast-cancer development. Alterations in a number of genes have been linked to hereditary breast cancer including *BRCA1*, *BRCA2*, *p53*, Ataxia telangiectasia mutated, PTEN, and Adenomatous polyposis coli. Inherited mutations in the breast cancer susceptibility genes, BRCA1 and BRCA2, are implicated in 40–50% of women with a familial history of breast cancer and have been reported to increase a woman's lifetime breast-cancer risk to as high as 85% [1,2]. BRCA2 mutations have been associated with a wide spectrum of disease outcomes including male breast, ovarian, prostatic, colon, pancreatic, and stomach cancers [3]. Studies of the BRCA2 999del5 mutation in the Icelandic population suggest the presence of modifier genes that can influence tumor phenotype [4]. However, the inheritance of a BRCA2 mutation in combination with a germline mutation in the breast-cancer–susceptibility gene BRCA1 has not been associated with more severe phenotypes [5,6].

Although the functions of the BRCA1 and BRCA2 genes have yet to be elucidated, there is evidence that they play a role in responses to DNA damage and the maintenance of genomic stability [7–13]. BRCA1 and BRCA2 interact directly in a complex with RAD51, suggesting interdependence in at least one DNA damage-repair pathway [13]. We and others have demonstrated that Brca1 and Brca2 expression patterns throughout embryonic and

L. Michelle Bennett and Kimberly A. McAllister contributed equally to the work presented in this report.

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Abbreviations: E, embryonic day; 129, 129/SvEv; BALB/c, BALB/cJ; ES cell, embryonic stem cell; PCR, polymerase chain reaction; neo, neomycin resistance; RT, reverse transcription.

mammary-gland development and in adult tissues are very similar, although not identical [14,15]. These similar expression patterns may or may not be the consequence of direct interactions between factors that control the expression of these two genes.

Most targeted genetic mutations described to date in the Brca1 and Brca2 loci cause embryonic lethality when present in the homozygous state [16]. The age of embryonic lethality in several Brca1- and Brca2-null embryos is dependent on which portion of the gene is targeted for disruption [17]. Targeted disruption of Brca1 in the 5′ region of the gene causes embryonic lethality from embryonic day (E) 4.5 to E7.5 [18,19]. A Brca1 mutation causing an in-frame exon 11 deletion can extend survival beyond E10.5 [20]. Similar observations have been made with Brca2 mice that target the 5′ end of exon 11. Targeted disruption 5′ of the well-conserved BRC repeats in exon 11 results in embryonic lethality when two mutant alleles are inherited [21–23]. However, retention of three or more of the BRC repeats, which bind Rad51, appears to be sufficient for survival of a small subgroup of homozygous-null animals to at least several months of age and is influenced by genetic background [24,25].

The targeted disruption of mouse genes has made it possible to study the modification of specific gene mutations in a controlled environment [17]. Several studies have demonstrated that combining a Brca1 or Brca2 alteration with *p53* or *p21* mutations extends embryonic survival. Brca1 and p53 or Brca1 and *p21* double-null embryos have survived to E9.5 [19,23], whereas Brca2/p53 double nullizygotes, on a mixed 129/SvEv (129) and C57BL/6 background, appear in most cases to be developmentally similar to or slightly more advanced than Brca2 mutants alone [23]. It is unclear how much of the phenotypic rescue described by introducing p53 or p21 in these studies is attributable to these specific targeted alterations as opposed to other genetic influences introduced by inbred strain backgrounds.

We introduced a single Brca2 gene mutation on the 129/SvEv (129) and BALB/cJ (BALB/c) genetic backgrounds. Although embryonic survival in Brca1- and Brca2-null mice can be influenced by an additional tumor suppressor gene alteration, the effects of genetic background alone have not been carefully investigated. We developed Brca2-deficient mice on a 129 background and established BALB/c inbred mice congenic for this alteration. The $129^{B2-/-}$ embryos express an embryonic lethal phenotype similar to previously reported Brca2-deficient mice with mutations introduced 5′ of the BRC repeats [21–23]. However, we demonstrate that the BALB/c inbred genetic background can prolong survival of homozygous-null embryos. The early embryonic death of Brca2-null embryos limits their use for studying loss of Brca1 and Brca2 function during neoplastic transformation. The extension of embryonic survivability enabled us to assess the interdependence of Brca1 and Brca2 by examining the expression pattern of Brca2 in $BALB/c^{B1-/-}$ embryos and Brca1 in $BALB/c^{B2-/-}$ embryos. We report that the BALB/c genetic background could enhance embryonic survivability in Brca2-null embryos and that Brca1 and Brca2 transcripts were expressed independently of one another.

MATERIALS AND METHODS

Development of Brca2-Deficient Mice

Brca2-deficient mice were generated by disrupting exons 10 and 11 with a pgkNEO cassette (Figure 1A). The pgkNEO gene replaced a portion of exon 10, intron 10, and the 5′ region of exon 11, extending from nucleotide 1745 to 2033 (accession number U89652) [26]. The targeting vector was linearized with SalI and electroporated (125 mFd, 0.36 kV; Bio-Rad gene pulser; Bio-Rad, Richmond, CA) into 129/Ola-derived BK-4 embryonic stem (ES) cells [27]. After positive and negative selection with geneticin (250 μg/mL; Gibco/BRL, Rockville, MD) and gancyclovir (2 μM; Roche, Hertfordshire, UK), three of 40 geneticin-resistant and gancyclovir-sensitive colonies were identified as correctly targeted by polymerase chain reaction (PCR), Southern analysis, and direct sequencing. One ES cell clone, 2S2, was used to generate several chimeric males by well-established techniques [28]. Initially, male chimeras were bred to wild-type C57BL/6 female mice, and DNA isolated from agouti tail biopsies was analyzed by PCR to identify offspring that carry the mutant Brca2 allele. The Brca2 mutation has been established on the C57BL/6N (Charles River Laboratory, Raleigh, NC) background by successive back crossing. Chimeric male mice were also bred directly to 129/SvEv (Taconic, Germantown, NY) and BALB/c (Jackson Laboratories, Bar Harbor, ME) females and back-crossed repeatedly to create inbred mouse strains congenic for the Brca2 mutation. The $BALB/c^{B2+/-}$ mice used in this experiment were from the seventh back-cross generation (>99% contribution from the BALB/c genetic background).

Mice

$BALB/c^{B1+/-}$ mutant mice were maintained by back crossing to wild-type BALB/c mice and have been described previously as $Brca1^{\Delta 223-763}$ mice [20]. The neomycin-resistance (*neo*) insertion in the $BALB/c^{B1+/-}$ mutant mice results in an alternatively spliced transcript that encodes an in-frame deleted Brca1 protein lacking amino acids 223–763 from exon 11. DBA/2J mice were purchased from Jackson Laboratories. All mice were group housed in plastic cages with pressed wood-chip bedding.

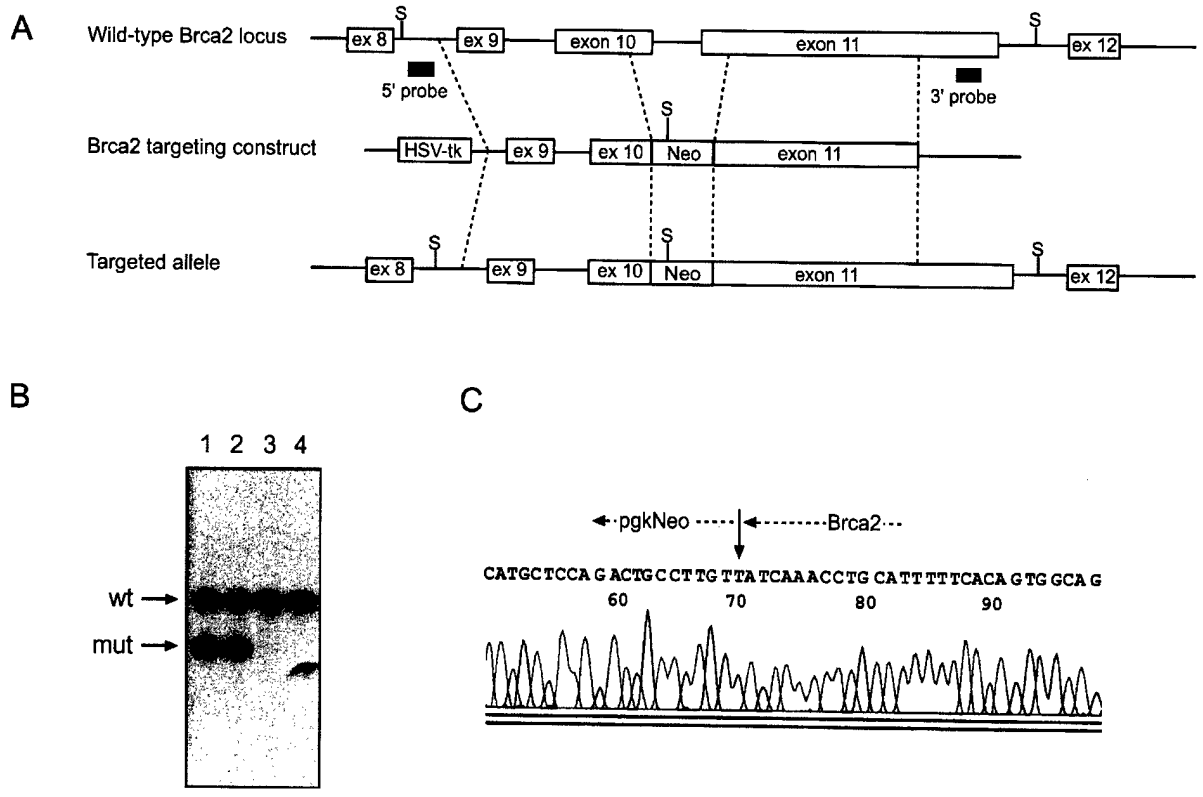

Figure 1. (A) Brca2 targeting strategy. The exon 10 and 11 region of the mouse Brca2 gene was disrupted by homologous recombination by using a targeting vector containing the selectable pgkNeo and HSV-tk genes. The pgkNeo gene replaced a portion of exon 10/11, causing a shift in the *Spe* I restriction enzyme pattern from 8.5 kb for the wild-type to 6 kb and 2 kb because of the presence of an additional SpeI site in the Neo gene. Homologous recombination was detected by both PCR and Southern blot analysis by using the SpeI restriction enzyme digest and the 3′ and 5′ probes. (B) Southern blot analysis of the DNA isolated from four agouti offspring of a chimeric Brca2-mutant male. Digestion with the restriction enzyme SpeI permits detection of an altered DNA digest pattern in two offspring (lanes 1 and 2) that have inherited the targeted Brca2 allele and two wild-type offspring (lanes 3 and 4) when hybridized with a 3′ exon 11 probe. (C) Direct sequencing of an RT-PCR product from BALB/$c^{B2-/-}$ mouse testis RNA. Arrow indicates *Brca2/neo* junction.

Animals had access to an NIH-31 diet (18% protein, 4% fat, and 5% fiber; Zeigler Bros., Gardeners, PA) and water ad libitum.

Genotyping by PCR and Southern Analysis

DNA from ES cells, tail biopsies, whole embryos, or day-old pups was isolated after overnight digestion with proteinase K at 55°C by phenol/chloroform extraction using serum separation tubes (Becton-Dickinson, Franklin Lakes, NJ). The wild-type Brca2 allele is detected by using the exon 11–specific primers KMF10 (5′-CTGAAGAGCCATCCTTGACC) and KM8R (5′-TCACTGTTCCCATCTGATTTC), which yield a 980-bp PCR product. The Brca2 Δ10/11 mutant allele is identified by PCR by using primers TkNeoF3 (5′-GAAGGATTGGAGCTACGGG) and JMR2 (5′-CTGAAGGAAGACATGCTGAAA), which yield a 300-bp product. Both Brca2 genotyping reactions are performed under the following conditions: denature at 94°C for 2 min followed by 30 cycles: 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and 72° for a 7-min extension.

The wild-type Brca1 allele is identified by using primers MBF3 (5′-GCACATTTATTACAGAACCAC) and MBR4 (5′-ACTTCCTCCTCAGCCTATTTTT) from exon 11 that yield a PCR product of 390 bp. The targeted Brca1 allele is identified by using the primers TkNeoF3 and MBR11 (5′-TTAAGCGCGTGTCTCAAGG), which yield a product of 550 bp. Both Brca1 primer pairs are used under the following conditions: denature at 94°C for 2 min, followed by 35 cycles: 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, with a final 8-min extension at 72°C.

The Brca2 Δ10/11 mutant allele was also detected by Southern blot analysis. Isolated DNA was digested with SpeI (New England Biolabs, Beverly, MA), resolved on a 0.8% agarose gel, and transferred to GeneScreen Plus (NEN Life Sciences Products, Boston, MA). Filters were hybridized with a 5′ mouse probe outside the targeting construct (Figure 1A) and generated by using the primers KMF22 (5′-CCCCAGCTAGCCTGAATTTT) and KMR9 (5′-CTTCTTGCTGGTTTTTGTTTTC).

Embryo Collection

Sexually mature adult female $129^{B2+/-}$ and BALB/$c^{B2+/-}$ mice were superovulated with 5 IU of

pregnant mare serum gonadotropin (Sigma, St. Louis, MO) followed by 5 IU of human chorionic gonadotropin (Sigma) 48 h later and were then mated with $129^{B2+/-}$ and BALB/$c^{B2+/-}$ males, respectively. E8.5, E10.5, and E11.5 embryos or deciduas were collected from timed-pregnant mice and fixed in 10% neutral buffered formalin for 2–24 h or frozen at −70°C for genotypic analysis. Sufficient tissue was not always available for the extraction of DNA and RNA. Female BALB/$c^{B1+/-}$ mice were also superovulated and mated with BALB/$c^{B1+/-}$ males to generate Brca1 embryos of all genotypes. E10.5 embryos or deciduas were subsequently collected from these timed-pregnant BALB/$c^{B1+/-}$ females and fixed as described earlier.

Reverse Transcription (RT)–PCR

RNA was extracted from mouse testes or whole embryos with Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) as directed. Frozen tissue samples were finely ground with mortar and pestle in liquid nitrogen. One milliliter of Tri-Reagent was added per 50–100 mg of tissue, the samples were homogenized, and RNA was isolated. The cDNA was generated by RT from the mRNA using Superscript II-RT (Boerhinger Mannheim, Indianapolis, IN). The Brca2 cDNA was amplified by an exon 10–specific forward primer KCF1 (5′-AGGACAGCATTTTGAATCACT) and a neo-specific reverse primer KCR1 (5′-GGTGGATGTGGAATGTGT). This RT-PCR product was gel isolated and sequenced directly with an internal reverse *neo* primer (5′-AGACGTGCTACTTCCATTTGT). Brca1 cDNA from Brca2-intercross progeny was amplified by RT-PCR with the following primer pair that spanned from exon 6 to exon 7 of Brca1: MBF29 (5′-GGGAAGCACAAGGTTTAGTCA) and MBR10 (5′-GGTGGCATTTCCAGGTTC).

In Situ Hybridization

Brca1 and Brca2 digoxigenin-labeled probes were generated as described by Blackshear et al. [14] except that the transcription reactions were performed with the Maxiscript T3/T7 kit (Ambion, Austin, TX). Paraffin-embedded sections were hybridized and washed at 60°C. Whole-embryo in situ hybridization was performed essentially as described by Wilkinson [29]. After fixation, the embryos were prehybridized for 1 h at 63°C and then hybridized overnight to either a Brca2-antisense or a Brca2-sense digoxigenin-labeled probe. After hybridization, the embryos were taken through a series of stringent 63°C washes (50% formamide, 0.75 M sodium chloride, and 0.075 M sodium citrate). The embryos were then treated with ribonuclease A to remove unbound single-stranded probe. The embryos were incubated with 10% sheep serum for 60–90 min and then incubated overnight at 4°C with antidigoxigenin antibody conjugated to alkaline phosphatase. The embryos were incubated with nitroblue tertrazolium/5-bromo-4-chromo-3-indolyl phosphate (Boehringer Mannheim) in the dark to develop the alkaline phosphatase histochemical reaction.

RESULTS

Genotype and Transcript Analysis in Brca2-Deficient Mice

The genotypes of agouti offspring resulting from crosses between chimeric males and C57BL/6 females were determined by Southern analysis (Figure 1B) and PCR (data not shown). RT-PCR analysis was performed on BALB/$c^{B2+/-}$ testis RNA because Brca2 RNA is abundantly expressed in this tissue [14,15,26]. A 550-bp fragment was generated from the BALB/$c^{B2+/-}$ samples with primers specific for Brca2 exon 10 and the *neo* gene. Direct sequencing of this PCR product indicated that the *Brca2/neo* fusion transcript was expressed (Figure 1C). The putative Brca2 protein is truncated at codon 569, with 75 additional amino acids contributed by the pgkNeo cassette. Repeated attempts to visualize a truncated transcript by northern blot analysis were unsuccessful, presumably due to unstable mRNA.

Phenotypic Evaluation of Brca2-Deficient and *Brca2*-Null Mice

Mendelian inheritance of the Brca2 mutation was studied by intercrossing male and female Brca2 heterozygous mice, with a mixed 129 × C57BL/6 background, to generate Brca2-null mice. Genotypic analyses by Southern blotting and PCR amplification failed to identify a single Brca2-null animal from 142 intercross offspring examined at weaning (Table 1). Mice heterozygous for a germline Brca2 mutation did not display any overt phenotypic abnormalities compared with their wild-type littermates at birth, weaning, or as adults. Female and male Brca2-heterozygous mice were able to breed normally. Females carried their pups to term and did not exhibit difficulty with lactation. We did not observe an increased incidence of spontaneous tumors in $129^{B2+/-}$ virgin females versus wild-type littermates up to 2 yr of age.

Genetic Modification of Embryonic Survivability

Genetic background has been determined to influence phenotype and survival of mutants in several gene knockout mouse models [30]. Brca1-null offspring on a mixed 129 × DBA/2 × C57BL/6 genetic background survive up to E13.5 [20], which is several days beyond that described for other Brca1-null mice. This altered phenotype may be the consequence of this specific Brca1 mutation that yields an in-frame deletion of exon 11 (Δ223–763) or, alternatively, may be enhanced by the presence

Table 1. Genotypes of Brca2-Intercross Progeny on Different Inbred Genetic Backgrounds

Strain background	Time point	Brca2 genotype		
		+/+	+/–	–/–
($C57^{B2+/-} \times 129^{B2+/-}$) F2	3 wk	65	77	0
(DBA/2 × $C57^{B2+/-}$) F2	1 d	6	19	0
(DBA/2 × $129^{B2+/-}$) F2	1 d	4	17	0
(DBA/2 × $C57^{B2+/-}$) F1 × $129^{B2+/-}$	1 d	13	12	0

of the DBA/2 genetic background. Thus, we evaluated survival to birth of Brca2-null mice by using several combinations of the DBA/2 with 129 and/or C57BL/6 genetic backgrounds. Seventy-one offspring from the following crosses were genotyped at 1 d of age: (DBA/2 × 129 $^{B2+/-}$)F2, (DBA/2 × C57BL/$6^{B2+/-}$)F2, and (DBA/2 × C57BL/$6^{B2+/-}$)F1 × $129^{B2+/-}$. As shown in Table 1, the incorporation of the DBA/2 genetic background did not prolong the survival of Brca2-null offspring to birth.

The embryonic lethal phenotype was studied in Brca2-null mice on the 129 genetic background. Intercrosses between male and female $129^{B2+/-}$ mice generated 22 E8.5 embryos, but genotypic analysis of the embryos was not completed because of the large number of reabsorbed fetuses observed at this time point. The $129^{B2+/-}$ intercrosses generated 35 E10.5 embryos, 11 of which were dissected out of the decidua or the yolk sacs and genotyped. Only heterozygotes and wild types were present at E10.5. Thus, $129^{B2-/-}$ embryos die and are largely reabsorbed before E8.5, which is consistent with previous reports of Brca2-deficient mice on a mixed C57BL/6 and 129 genetic background [21–23].

In contrast, when the Brca2 mutation was placed on a BALB/c genetic background, the day of embryonic lethality in the Brca2-null mice was extended to approximately E10.5. Ten E8.5 embryos were generated from BALB/$c^{B2+/-}$ intercross matings. Seven of 10 E8.5 embryos were genotyped, and the remaining three were either partly or completely reabsorbed. PCR analysis conclusively identified three BALB/$c^{B2-/-}$ embryos and four BALB/$c^{B2+/-}$ embryos (Figure 2). Genotyping was performed on one sample that was partly reabsorbed, but the PCR product was probably due to maternal contamination (Figure 2, lane 4). BALB/$c^{B2-/-}$ E8.5 embryos were grossly smaller, developmentally delayed, and resembled E6–7 embryos (Figure 3). Twenty-six yolk sacs and/or embryos were isolated from intercrosses

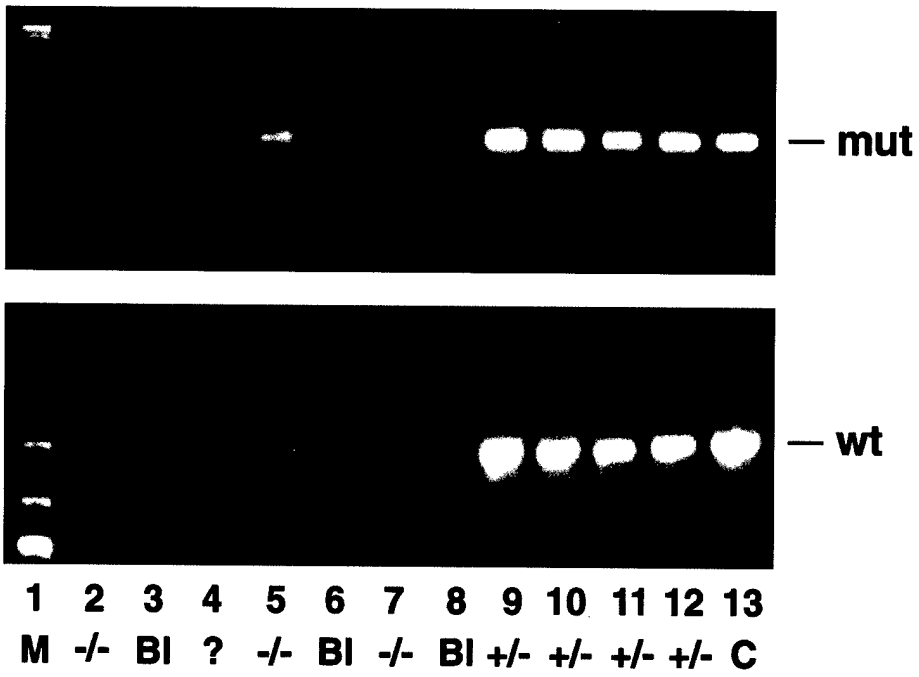

Figure 2. Genotypic analysis of DNA extracted from eight intercross E8.5 BALB/c^{B2} embryos. The upper gel contains PCR products using primers that amplify the mutant allele. The lower gel contains PCR products generated with wild-type–specific primers. Each lane represents an individual embryo. No samples were loaded in lanes 3, 6, and 8. Lane 1: DNA MW marker. Lanes 2, 5 and 7: BALB/$c^{B2-/-}$ embryo. Lane 4: inconclusive result. Lanes 9–12: BALB/$c^{B2+/-}$ embryos. Lane 13: control adult BALB/$c^{B2+/-}$ DNA.

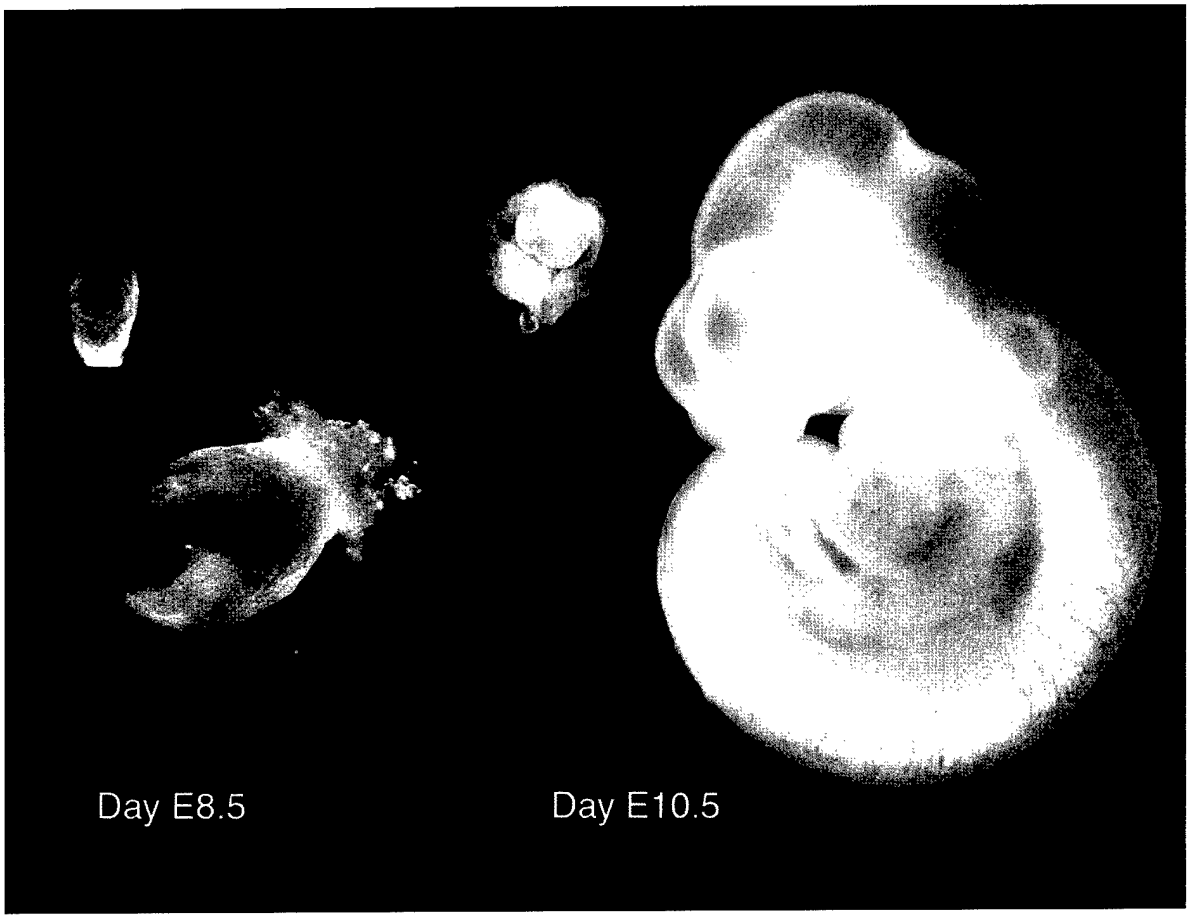

Figure 3. Growth inhibition of BALB/$c^{B2-/-}$ embryos at E8.5 and E10.5. The E8.5 growth-retarded BALB/$c^{B2-/-}$ embryo is compared with a normal BALB/$c^{B2+/-}$ littermate. The Brca2 (–/–) embryo is still in an egg-cylinder stage. The Brca2 (+/–) embryo has approximately four to five somites and well-developed ectoplacental cone and yolk sac. The E10.5 growth-retarded BALB/$c^{B2-/-}$ embryo is compared with a morphologically normal wild-type littermate. The Brca2 (–/–) embryo is developmentally delayed, with an open cranial neural tube characteristic of a normal E8.5 embryo.

at E10.5. The yolk sacs and/or embryos were genotyped by PCR and confirmed that all morphologically normal embryos were of the heterozygous or wild-type genotypes. The one confirmed BALB/$c^{B2-/-}$ embryo that survived until E10.5 was developmentally delayed and smaller than its heterozygous littermates. The cranial neural tube remained open, characteristic of an E8.5 embryo. However, the turning process was completed and the second brachial arch and the heart were prominent, indicating some development beyond the E8.5 time point. Three additional embryos displayed similar developmental delays, but confirmation of genotype was hindered by maternal tissue contamination. No embryonic tissue was found in five reabsorption sites. At the E11.5 time point, 10 morphologically normal and two developmentally delayed yolk sacs were dissected. There were three completely reabsorbed sites that could not be dissected. The 10 morphologically normal embryos were genotyped as wild types or heterozygotes. The confirmation of genotype for the two developmentally delayed embryos was not possible because of contaminating maternal tissue. Developmentally, these presumed mutant embryos were characteristic of normal E8.5 embryos, similar to that observed at the E10.5 time point.

The extension of Brca2-null embryo survival on the BALB/c genetic background enabled us to address whether transcriptional regulation of the Brca1 and Brca2 genes was interdependent. Brca1 expression was evaluated by RT-PCR analysis of mRNA isolated from E8.5 BALB/$c^{B2-/-}$ embryos. These studies demonstrated that Brca1 expression in BALB/$c^{B2-/-}$ E8.5 embryos was comparable to age-matched wild-type BALB/$c^{B2+/-}$ embryos (Figure 4).

In addition, we examined the timing of embryonic lethality and the expression of Brca2 in Brca1-null embryos on the BALB/c genetic background. Forty-two embryos were generated from BALB/$c^{B1+/-}$ intercrosses, and 19 viable progeny were examined. Genotypic analysis showed two BALB/$c^{B1-/-}$, seven BALB/$c^{B1+/+}$, and 10 BALB/$c^{B1+/-}$ offspring. The BALB/$c^{B1-/-}$ embryos were grossly

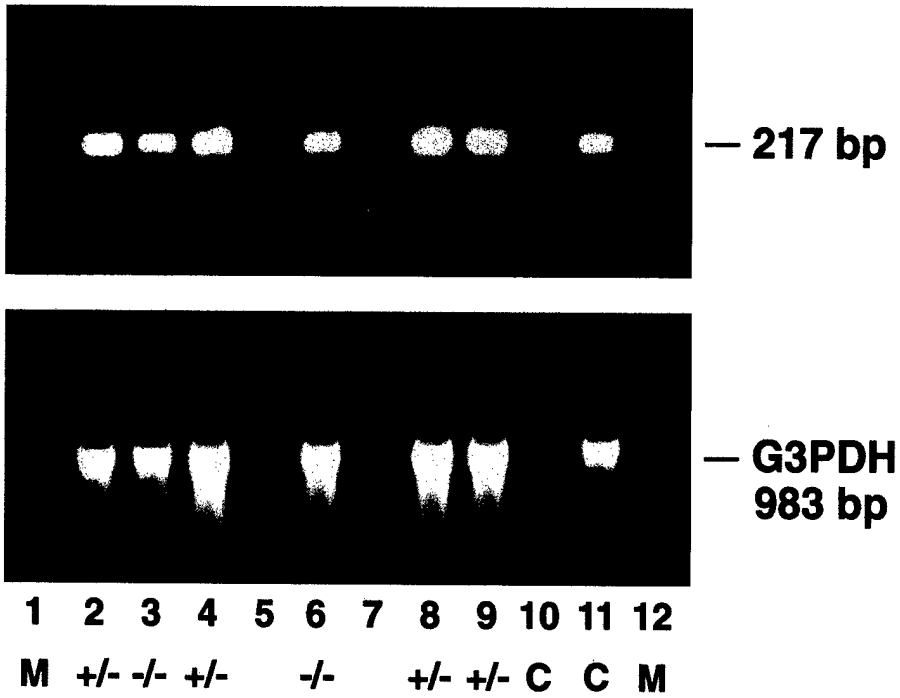

Figure 4. Analysis of Brca1 expression in the E8.5 BALB/c^{B2} embryos from Figure 3 by RT-PCR. The genotypes are indicated. The upper gel shows Brca1 cDNA (217 bp) amplified with a primer pair that spans exons 6 and 7. G3PDH, used as an internal control, is shown on the lower gel. Lanes 1 and 12: DNA MW markers. Lanes 2–4, 6, 8, and 9: amplification of Brca1 cDNA. Lane 11: positive control. Lanes 5 and 7: no Brca1 cDNA was amplified because of the minimal amount of embryo tissue available. Lane 10: negative control.

smaller and developmentally delayed. The E10.5 Brca1-null embryos had completed the turning process characteristic at this stage of development. However, the open cranial neural tube was characteristic of E8–8.5 embryos. These observations are comparable to results reported by Gowen et al. [20] with Brca1-null mice on a mixed 129, DBA/2, and C57BL/6 genetic background. Thus, we did not observe a dramatic alteration in the timing of embryonic lethality in BALB/$c^{B1-/-}$ embryos.

Brca2 expression was examined in E10.5 BALB/$c^{B1-/-}$ embryos. In situ hybridization analysis detected widespread Brca2 expression in Brca1-null embryos that was qualitatively comparable to the levels of Brca2 expression in age-matched BALB/$c^{B1+/+}$ embryos (Figure 5). The most intense localization of Brca2 mRNA was in the neuroepithelial cells lining the neural tube and was similar between wild-type and null embryos (data not shown). Neuroepithelial cells comprised the most active proliferating population, correlating with rapid brain development, in E8–10 embryos.

DISCUSSION

This study demonstrates that the BALB/c genetic background can modify survival of *Brca2*-null mouse embryos. Previous reports have associated embryonic survival with the location of mutations within the gene but have not shown an effect due to variation of genetic background alone. The $129^{B2-/-}$ embryos were presumed to have died before E8.5 because $129^{B2+/-}$ intercrosses had few viable progeny, as indicated by the large percentage of reabsorbed fetuses. This observation is consistent with other reports of Brca2 mutant mice in which the targeted alteration was 5′ of the BRC repeat region [21–23]. In contrast, matings between BALB/$c^{B2+/-}$ mice generated BALB/$c^{B2-/-}$ embryos that survived to E10.5. Thus, the BALB/c genetic background extends survival of *Brca2*-null embryos that inherit an alteration in exons 10 and 11 in Brca2.

Genetic background has been shown to influence phenotype and survival of mutants in a variety of gene knockout animal models [30]. For example, the BALB/c genetic background has a dramatic effect on the normal development of *p130* knockout mice [31]. Likewise, the timing of embryonic lethality of PTEN knockout embryos is modified by the CD-1 genetic background [32,33]. Epidermal growth factor receptor knockout mice with a 129 inbred background die during mid-gestation but survive to birth or longer with mixed inbred or outbred genetic backgrounds [34,35]. Thus, embryonic lethality for a variety of gene defects can be modified by allelic differences contributed by various inbred strain backgrounds.

We have demonstrated that modifiers in the BALB/c genetic background can prolong the survival of Brca2-null embryos. Variation of disease onset and severity has been reported for women with

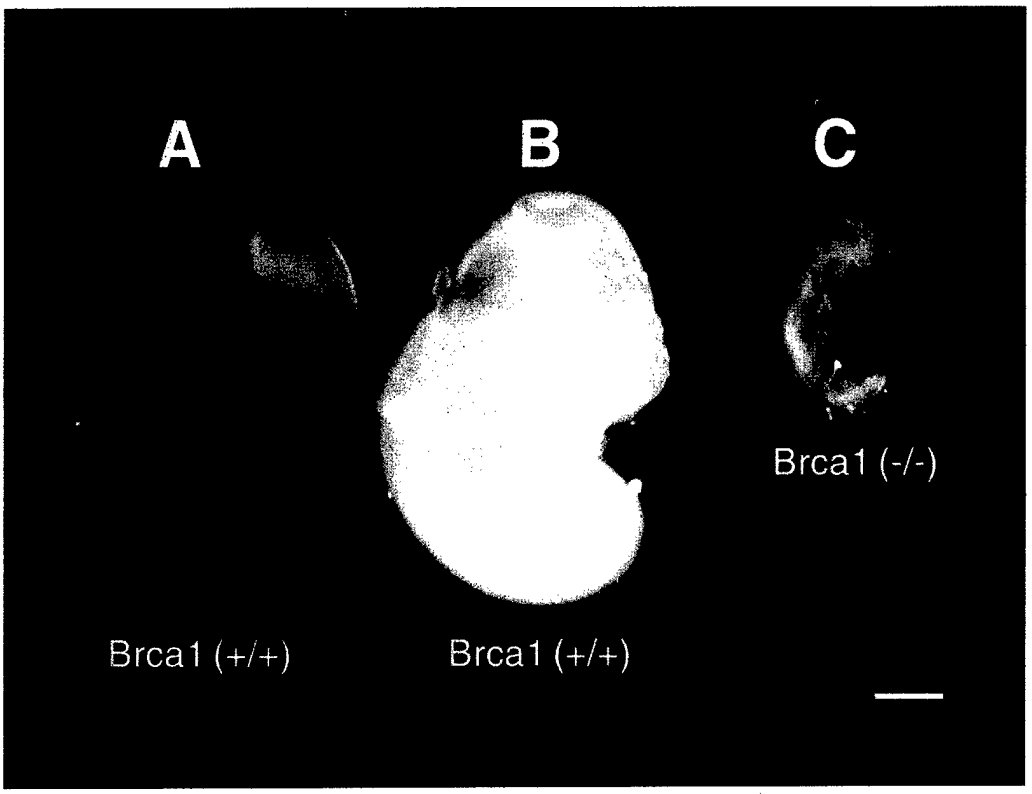

Figure 5. In situ hybridization analysis of Brca2 expression in E10.5 BALB/$c^{B1+/+}$ and BALB/$c^{B1-/-}$ embryos: BALB/$c^{B1+/+}$ whole embryos were hybridized with cRNA digoxigenin-labeled Brca2 antisense (A) and sense (B) probes. (C) BALB/$c^{B1-/-}$ embryo hybridized with cRNA digoxigenin-labeled antisense probe. Bar = 1 mm.

various mutations in BRCA1 and BRCA2 [4,36]. Penetrance variations in different populations suggest that genetic modifier loci or environmental factors must modulate the effect of BRCA1 or BRCA2 inactivation [1]. The inheritance of mutations in both BRCA1 and BRCA2 does not appear to affect severity or latency of disease outcome in humans [5,6], as might be expected if the genes directly interacted. However, modifier genes are strongly implicated by the various disease phenotypes that have been observed in individuals with identical BRCA2 mutations [4]. Certainly, there may be highly prevalent low-penetrance susceptibility genes that have a more profound effect on breast-cancer development in the general population than BRCA1 or BRCA2 mutations do [37,38]. Such genes may also modify breast-cancer risk in BRCA1 and BRCA2 mutation carriers, but the limitations of human linkage analysis makes identifying those genes very challenging.

Radiation-induced DNA damage is repaired less efficiently in cells from BALB/c than in cells from C57BL/6 mice. Chromosomal aberrations persist for up to 28 population doublings in irradiated BALB/c mammary epithelial cells, whereas similarly treated C57BL/6 cells repair damage within six population doublings [39]. Perhaps this increased tolerance for DNA damage in BALB/c mice is related to the prolonged embryonic survival of BALB/$c^{B2-/-}$ mice compared with other strains. In addition, a Brca2 mutation on the BALB/c genetic background may result in more dramatic phenotypic outcomes in the adult mammary gland as a result of inefficient DNA damage repair and a predicted tissue specificity.

This study also demonstrated that Brca1 mRNA was expressed in Brca2-null embryos and Brca2 mRNA was expressed in Brca1-null embryos. Expression levels of the null embryos were qualitatively comparable to those of wild-type and heterozygous littermates, although small quantitative differences in expression cannot be excluded. Thus, Brca1 and Brca2 transcripts are expressed independently of each other. If the regulation of either of these genes were dependent on the other, dysregulated expression would be expected in the gene-deficient embryos. Our results support those of a report by Suzuki et al. [22] who used *Brca1*-null mice with a targeted disruption of exons 5 and 6 and *Brca2*-null mice with disruption of exons 10 and 11 to study Brca2 and Brca1 gene expression, respectively. In the mouse embryo, adequate proliferation to sustain normal development appears to require the expression of both Brca1 and Brca2. Mice that inherit mutations in both Brca1 and Brca2 genes do not display more severe embryonic lethality, which might be expected if the effect of the losses were

additive [23]. Our investigation was limited to mRNA expression in embryos and did not address potential interactions between the Brca1 and Brca2 proteins. Because these gene products form a complex in vitro [13], it is possible that the functions of these proteins require a mutual interaction that permits normal cellular proliferation such that dysregulation of either Brca1 or Brca2 can contribute to neoplastic development.

We have described a substantial extension in the survival time of Brca2-null embryos on the BALB/c genetic background. These results suggest the presence of modifier loci that could be mapped in these mice. Future studies will evaluate the influence of the BALB/c genetic background on mammary-gland morphogenesis and neoplastic development in Brca2-deficient mice. Identification of such genetic modifiers in mice may permit the identification of human orthologues that could be evaluated for their role in breast-cancer susceptibility.

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Spatial and Temporal Expression of the Cre gene under the Control of the MMTV- LTR in Different Lines of Transgenic Mice

Running title: Expression of MMTV-Cre transgenic mice

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Abstract

Cre-loxP based gene deletion approaches hold great promise to enhance our understanding of molecular pathways controlling mammary development and breast cancer. We reported earlier the generation of transgenic mice that express the Cre recombinase under the control of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR). These mice have become a valuable research tool to delete genes specifically in the mammary gland, other secretory organs, and the female germline. We have now characterized in depth the expression of the MMTV-Cre transgene using the ROSA26-lox-Stop-lox-LacZ reporter strain to determine the temporal and spatial activation of Cre on the level of single cells. Our results show that MMTV-mediated Cre-activation is restricted to specific cell types of various secretory tissues and the hematopoietic system. Secondly, the timing of Cre expression varies between tissues and cell types. Some tissues express Cre during embryonic development, while other selected cell types highly activate Cre around puberty, suggesting a strong influence of steroid hormones on the transcriptional activation of the MMTV-LTR. Thirdly, Cre expression in the female germline is restricted to individual mouse lines and is therefore dependent on the site of integration of the transgene. Information provided by this study will guide the researcher to those cell types and developmental stages at which a phenotype can be expected upon deletion of relevant genes.

Key words: MMTV-LTR, Cre, transgenic mice, mammary, ROSA26

Introduction

Over the past decade, the gene targeting technique has permitted an unparalleled insight into genetic pathways involved in mouse development and tumorigenesis. Despite these achievements, the role of many genes in development and disease remains elusive, since their deletion from the murine genome is either lethal or does not mimic closely the progression of the disease in humans. Furthermore, many human disorders are the result of sporadic, acquired mutations in a limited number of cells that are surrounded by normal tissues. Hence, targeting gene deletions or modifications precisely to specific cell types at a distinct developmental stage is essential to establish high fidelity mouse models for human diseases. Such defined mutations can now be modeled in a temporal and spatial fashion using the Cre-lox technology.

The Cre-lox technology is a binary system, where the Cre recombinase catalyzes the deletion of a DNA fragment between two directly orientated *loxP* sites (Hoess *et al.*, 1984; Hoess *et al.*, 1987). LoxP recognition sites are inserted around the regions to be deleted using homologous recombination, thus creating an allele, which is flanked by *loxP* sites (a floxed allele). The temporal and spatial excision of the floxed allele is achieved by expressing the Cre enzyme under a tissue-specific promoter in the same mouse (Nagy, *et al.*, 2000).

MMTV-Cre transgenic lines were generated in an attempt to obtain consistently high expression of Cre recombinase in mammary epithelium and this has now been established. An analysis of recombination in double transgenic mice carrying a reporter transgene and the MMTV-Cre was initially performed in an attempt to determine the expression pattern of Cre in these animals (Wagner *et al.*, 1997). This study, as well as RT-PCR assays, revealed that Cre was active in many organs of MMTV-Cre mice, although these studies did not permit an analysis at the level of single cells. There was an initial uncertainty as to whether these mice could be beneficial for a temporal and spatial gene deletion approach. However, the MMTV-Cre mice have now been used successfully by many laboratories. The lack of embryonic lethality of offspring from crosses of MMTV-Cre lines with more than a dozen floxed genes has allowed the development of more appropriate, tissue-specific animal models for several human diseases. The inactivation of several genes, including *Brca1*, which had been deleterious in conventional gene deletion approaches, has been successfully performed through a deletion generated by this conditional knockout approach (Xu *et al.*, 1999).

The availability of the ROSA26 LacZ reporter strain has allowed the identification of Cre expression at a cell-specific level (Soriano, 1999). We have used these ROSA26 LacZ reporter mice to reinvestigate the expression pattern of the MMTV-Cre transgenic lines on a single cell level. The MMTV-Cre mice have now been distributed to more than 20 laboratories worldwide and are now available at the Jackson Laboratory (Bar Harbor, Maine). The results of the expression patterns observed for the MMTV-Cre transgenic lines using the ROSA26 LacZ reporter strain should be helpful for directing investigators

using these animals to identify the specific cells in which Cre is expressed and the precise timing of Cre activation.

Material and Methods

Lines A and D carrying the MMTV-Cre transgene (Wagner *et al.*, 1997) were crossed into the ROSA26 LacZ reporter strain (Soriano, 1999). Offspring of these crosses, which carried both the Cre transgene and the ROSA26 LacZ transgene, were analyzed for LacZ expression along with the offspring carrying only the ROSA26 transgene alone (negative control). Mice carrying the Cre transgene gene were identified by PCR using the following forward and reverse primers: 5'GCCTGCATTACCGGTCGATGC3' and 5'CAGGGTGTTATAAGCAATCCCC3'. The ROSA LacZ locus was identified by PCR using the following forward and reverse primers: 5' GATCCGCGCTGGCTACCGGC3' and 5'GGATACTGACGAAACGCCTGCC3'. All tissues were fixed for 1-2 hours in 2% paraformaldehyde, 0.25% glutaraldehyde, .01% NP-40 in PBS and stained for β-galactosidase activity (1 mg/ml X-gal, 30mM $K_3Fe(CN)_6$, 30 mM $K_4Fe(CN)_6 \cdot 3H_20$, 2mM $MgCl_2$, 0.01%Na-deoxycholate, 0.02% NP-40, 1x PBS) overnight at 30°C. Mammary glands were postfixed in 10% formalin, dehydrated to 100% EtOH, and placed overnight in xylene before whole mount analysis. All other tissues were dehydrated to 70% EtOH, embedded in paraffin, sectioned, and counterstained with Nuclear Fast Red. Both of these MMTV-Cre transgenic lines are now available from the Jackson Laboratory (line A: Stock # 003551, B6129-Tgn(MMTV-Cre)1Mam; line D: Stock #

003553, B6129-Tgn(MMTV-Cre)4Mam. All animals used in the described studies were treated humanely and in accordance with Public Health Service policies and federal regulations.

Results

The MMTV-Cre transgenic lines express the Cre gene under control of the MMTV-LTR (Wagner *et al.*, 1997). The MMTV-LTR has been used extensively to target different genes to mammary tissue. Since many of these studies focused on transforming genes and its effect on mammary epithelium, it was concluded that the MMTV-LTR is expressed preferentially in mammary tissue. However, previous studies did not provide clear information on the cell-specificity and temporal activation of the MMTV-LTR. We addressed these questions through an analysis of two independent transgenic strains (lines A and D) of mice that carry the MMTV-Cre transgene and the ROSA26 LacZ reporter locus. In ROSA26 LacZ reporter mice the lacZ gene within the ROSA locus is silent, but can be activated upon deletion of the lox stop sequence by Cre recombinase (Soriano, 1999). The ROSA26 promoter is expressed in essentially all embryonic and adult mouse tissues. Cre-mediated activation of the lacZ gene will therefore mark an expressing cell and all its descendants.

Expression during mammary gland development

Overall, the two transgenic lines investigated (lines A and D) displayed similar expression profiles although some differences were noted (Table 1). When the ROSA-lox-STOP-lox-LacZ reporter gene is activated in a cell by Cre-mediated recombination, this cell and all of its descendants will express beta-galactosidase and will stain blue with X-gal. All studies were performed comparing multiple sections from multiple animals that carried both the ROSA26 LacZ transgene and the MMTV-Cre transgene (positive sample) compared to mice that contained the ROSA26 LacZ transgene but not the MMTV-Cre transgene (negative control). Cre expression, as monitored by blue staining, was observed in ducts and alveoli in the mammary gland (Figure 1). Whole mount analyses demonstrated that mammary ductal cells in mice from line A had undergone recombination as early as day 6 after parturition (Fig. 1A). In contrast, line D displayed no recombination as observed by whole mount analyses in day 7-8 day-old pups or two-week-old animals. Staining in mammary tissue from the D line was first observed at 22 days of age and continued to show consistent expression from this timepoint onwards. By five weeks of age, both lines showed intense staining in most ducts and terminal endbuds, and by 6 weeks of age the entire ductal tree had undergone recombination in both lines (Fig. 1B and D). As the mammary gland develops, ductal structures elongate and branching occurs. Ductal elongation proceeds from the terminal end buds (TEBs), which consist of body cells and cap cells destined to become ductal epithelium and

myoepithelium. Both the body cells and CAP cells had undergone recombination in both lines (Figure 1C). Some lymphocytes in the lymph node of the mammary gland had also undergone recombination. Both lines showed extensive expression in epithelial and myoepithelial cells with little or no expression in stroma, fibroblasts, or adipocytes of the mammary gland. Although some mosaicism of staining of epithelial and myoepithelial cells was observed in adult animals (Figure 2 A-F), the majority of these cells expressed Cre recombinase.

During pregnancy the lobulo-alveolar mammary epithelium develops from a progenitor stem cell population. This involves extensive cell proliferation and differentiation. Whole mount analysis revealed extensive, although not complete recombination within the lobulo-alveolar compartment (Figure 1E and F). Line A (Figure 1G) exhibited less mosaicism than line D. Some variation was observed between individual mice. This mosaicism demonstrates that some alveolar progenitor cells did not express the MMTV-Cre transgene. At three and ten days of involution, most but not all of the ducts remained positively stained (data not shown).

MMTV-Cre expression is restricted predominantly to secretory cell types

The MMTV-LTR is active not only in mammary tissue but also in other secretory cell types. In our initial study of the MMTV-Cre mice (Wagner *et al.*, 1997), we used RT-PCR as well as a reporter construct to survey expression and concluded that limited recombination had occurred in every organ. We now analyzed a range of tissues from both lines of transgenic mice on a cellular level (Figure 3). These studies demonstrated

that in 3-month-old mice distinct cell types within several organs undergo MMTV-Cre mediated recombination. Both male and female mice at all ages in both lines showed moderate to intense lacZ staining of the submandibular gland (Figure 3A), submaxillary, and parotid glands in the salivary gland. Little recombination was observed in the sublingual gland. Cre-mediated deletion was also detected in secretory cells of the skin epithelium and in hair follicles (Figure 3B), Leydig cells (Figure 3C), epithelial lining of the vas deferens (Figure 3D), and seminal vesicles (Figure 3E). Although we observed some mosaicism, the vast majority of cells in these tissues had undergone a recombination event. In addition to these epithelial cells, we observed recombination in B- and T-cells (Figure 3G and H) and megakaryocytes of spleen in both lines (Figure 3H) and in erythroid cells (data not shown). Analysis of Ter119- positive erythroid cells revealed that a majority of the cells had undergone Cre-mediated recombination (Wagner *et al.*, 2000). Both lines also showed staining in acini but not islets of pancreas (data not shown).

Other tissues displaying limited Cre expression in 5- and 12-week-old animals from both lines were liver, trachea, and brain neurons. Little or no expression was ever observed in stomach, intestines, adrenal glands, kidney, lung, heart, skeletal muscle, or pituitary. In 3- and 6-month-old animals from line A, we observed additional staining in the bronchiolar epithelial cells, adrenal gland (focal areas of adrenal cortical cells) and numerous areas of female reproductive tract including the endometrial gland and luminal epithelium of the uterus and surface squamous epithelium of the vagina (data not shown). This staining was not observed in younger mice from line A or in mice from line D at any

age. Some staining was observed in the prostate for both strains at various ages but this staining was also observed in some control animals and therefore may not represent true positive expression of Cre in this tissue. We further investigated whether recombination can already be observed during fetal development. Recombination in the skin occurs already prior to embryonic day 11.5, as demonstrated by the blue embryos (Figure 3I) and is clearly visible at days 13.5 and 15.5 (Figure 3J and K).

Cre expression in oocytes

Unlike the MMTV-Cre line D, conditional alleles that pass through the female germline of MMTV-Cre line A are deleted in all tissues of the resulting offspring. This suggested that for line A, the integration site of the transgene permitted the MMTV-LTR to be active in oocytes during follicular development. Both 4- and 12-week old ROSA26 LacZ mice carrying the MMTV-Cre A transgene showed intense lacZ staining of some oocytes (Figure 3F) compared both to control littermates not carrying the Cre transgene and to ROSA26 LacZ mice carrying the MMTV-Cre D transgenic line. Staining was observed in some primordial and primary follicles with intense staining in the preantral and antral follicles compared to controls. This staining was present in viable oocytes and the cytoplasm of some degenerating oocytes. Although some staining was observed in the granulosa cells, thecal cells, interstitial cells, and corpora luteal cells of the ovary as well, this staining was also observed in some control samples and is therefore interpreted to be non-specific staining due to endogenous galactosidase activity.

Conclusion

These studies define the cell-specific expression pattern of the Cre gene under control of the MMTV-LTR in two independent lines of transgenic mice (Table 1). Consistent and mostly uniform recombination has been observed in mammary ductal epithelium, the acinar epithelium of the submandibular gland, skin, Leydig cells, seminal vesicles, megakaryocytes, B-cells, T-cells and hematopoietic cells. Both lines displayed specificity for epithelial cells in the mammary gland but the precise timing of Cre activation appeared to be slightly different for the two lines. In addition, Cre activity in oocytes was only observed in line A.

For conditional Cre-loxP studies, the timing of Cre activation in the particular transgenic line used can be critical for avoiding embryonic lethality inherent in the germline knockout of many genes and in avoiding non-specific phenotypes unrelated to the target tissue for the human disease one hopes to model. Precise activation of Cre can also be important for conditional studies to determine the precise timing of any treatments used for the conditional animals such as irradiation or carcinogen administration. The specificity of Cre activation observed in these studies in the mammary epithelial cells for both MMTV-Cre line D and MMTV-Cre line A makes this Cre transgenic animal an appropriate model to use when focusing on this key target cell type in the mammary gland.

Although expression of Cre in these MMTV-Cre lines is clearly not restricted solely to mammary epithelial cells, precise mammary gland phenotypes have been successfully generated with these mice. Conditional homozygous *Brca1* animals generated with the MMTV-Cre D line initially displayed a severe inhibition of mammary ductal branching (Xu *et al.*, 1999). These conditional *Brca1* mice went on to develop subsequent tumor formation after a long latency period (10-13 months of age) with a pathology similar to human breast cancer (Xu *et al.*, 1999). Our present study shows that Cre expression in the lobulo-alveolar compartment of the mammary gland exceeded 50% but exhibited mosaicism. Given that the majority of ducts did remain positively stained after lactation and involution in this present analysis, it is highly likely that Cre activation and subsequent inactivation of the conditional knockout gene would not be substantially disrupted with the remodeling of the gland during involution.

The differences observed in expression pattern between these two distinct lines can be exploited for slightly different uses by individual investigator's needs. The specific activation of the D line seems to coincide more closely with the initiation of puberty in the mammary epithelial cells. In general, this line might be more closely under the control of ovarian hormonal control as one might expect for a "classic" MMTV-driven transgene. The D line might therefore be more appropriate for use in the context of a conditional knockout where one wants precise inactivation of their gene to occur only with the initiation of ovarian function.

Females from the MMTV-Cre Strain A line which also carry a floxed gene have the particularly useful and unique feature of passing on a null allele to their offspring. This feature allows the investigator to use this unique line for generation of not only conditional but germline deletions of the gene of choice and several laboratories have recently utilized this approach (Loots *et al.,* 2000; Rucker *et al.*, 2000; Gérard *et al.,* 1999). This unique expression pattern in this line is probably due to the integration of the transgene into an as yet unidentified gene active in oocyte development. This has been confirmed in this study by the recombination observed in oocytes from line A, but not in line D. Despite the fact that staining in the oocytes appears mosaic, we have never observed an unrecombined allele when passed through the female germline of an MMTV-Cre line A mouse. It is possible that not all recombined cells always stain for galactosidase activity due to staining artifacts or perhaps due to a ROSA promoter expression profile which is not completely ubiquitous. We have not excluded the possibility that additional recombination occurs at the time of ovulation in this line as well but the activation of Cre appears to occur at least to some extent very early from line A in the oocytes.

In addition to the distinct oocyte expression profile, line A exhibited several other features unique from line D. Although both lines should be appropriate for inactivating genes in mammary epithelial cells, line A may be especially useful for studying the influence of genes on the development of the primary duct of the mammary gland prior to puberty since activation of Cre in this tissue appears so early in this line. Some differences in the Cre expression pattern in line A appear to vary with age with relatively

wider expression in tissue types displayed for older animals. This suggests that activation in some tissues might be time-dependent in this line as well.

The easy availability of the ROSA26 LacZ reporter mouse and the MMTV-Cre Strain A used in combination with the conditional allele of the gene of choice will allow investigators to directly follow the precise cell type specificity of all alterations or phenotypes observed. Since limited mosaicism may influence these experiments in which cell survival molecules are deleted, the inclusion of a marker gene, such as the ROSA26 LacZ reporter mouse, in the experiment to mark those cells that have undergone deletion is highly recommended.

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Figure Legends

Table 1

Expression profile for the MMTV-Cre A and D transgenic lines.

***, high, **, moderate, *, low recombination efficiency; No, Cre-mediated recombination undetectable; pp, post-partum.

Figure 1

Mammary Epithelium at Different Developmental Stages

A) ducts, 6 day old female; B) 6 week old virgin; C) cross section through a terminal end bud (TEB); D) cross section through a duct; E) whole mount, lactation (line D); F) cross section through whole mount shown in E; G) whole mount, lactation (line A).

Figure 2

Variable Staining of Mammary Epithelium

A) photomicrography of a 5-week old mammary gland from a ROSA26 LacZ/MMTV-Cre D mouse. Low magnification of positively staining ducts. Arrows point to positively staining ducts. Arrowheads point to lymph nodes.

B) higher magnification of mammary gland with partial staining of epithelial and myoepithelial cells (arrow and arrowhead).

C), D) and E) differential staining of individual ducts.

F) littermate control. Arrows point to control stained ducts. Arrowhead points to a lymph node.

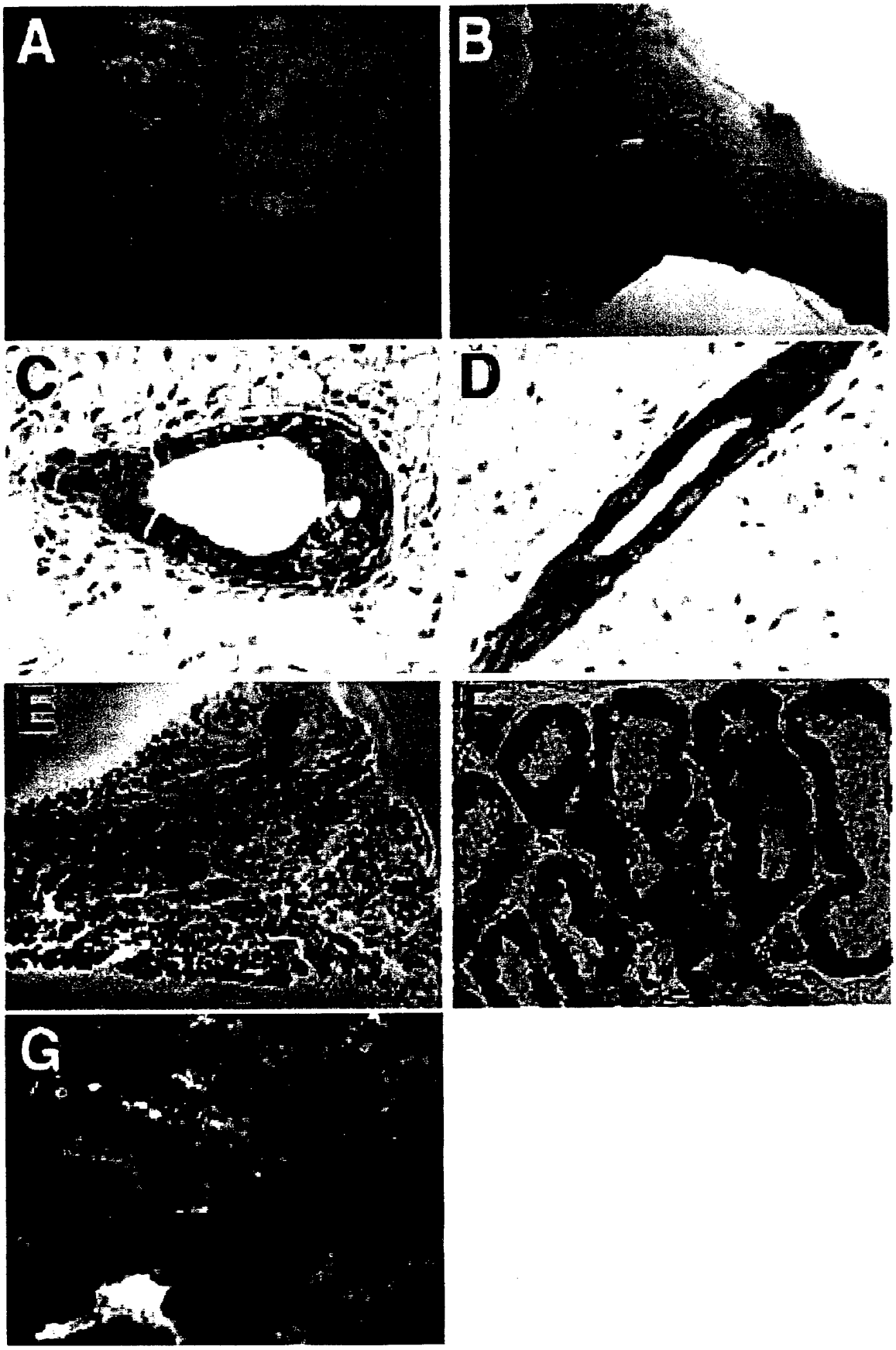
Figure 3

Organ Profile in MMTV-Cre Line A

A) submandibular gland; B) skin; C) cross section through adult testis (arrow points to Leydig cells); D) vas deferens; E) seminal vesicle (arrow points to an area of mosaic expression); F) ovary of an adult female (arrow points to the oocyte); G) thymus; H) spleen (arrow points to a megakaryocyte); I) day 11.5 fetus; J) day 13.5 fetus; K) day 15.5 fetus.

Table 1. Tissue/Cell-Type Expression Profile of two MMTV-Cre Transgenic Lines

	Line A	*Line D*
mammary epithelial cells	*** (>day 6 pp)	*** (>day 22 pp)
oocytes	***	No
salivary gland (epithelial cells)	***	***
skin (epidermis, hair follicles)	***	***
Leydig cells	**	**
vas deferens	**	**
seminal vesicles	***	***
B and T cells	***	***
megakaryocytes	**	***
erythroid cells	**	***
pancreas (acini)	**	**
liver	*	*
trachea	*	*
brain	**	**
bronchiolar epithelial cells	** (in adult only)	No
adrenal gland	** (in adult only)	No
female reproductive tract	** (in adult only)	No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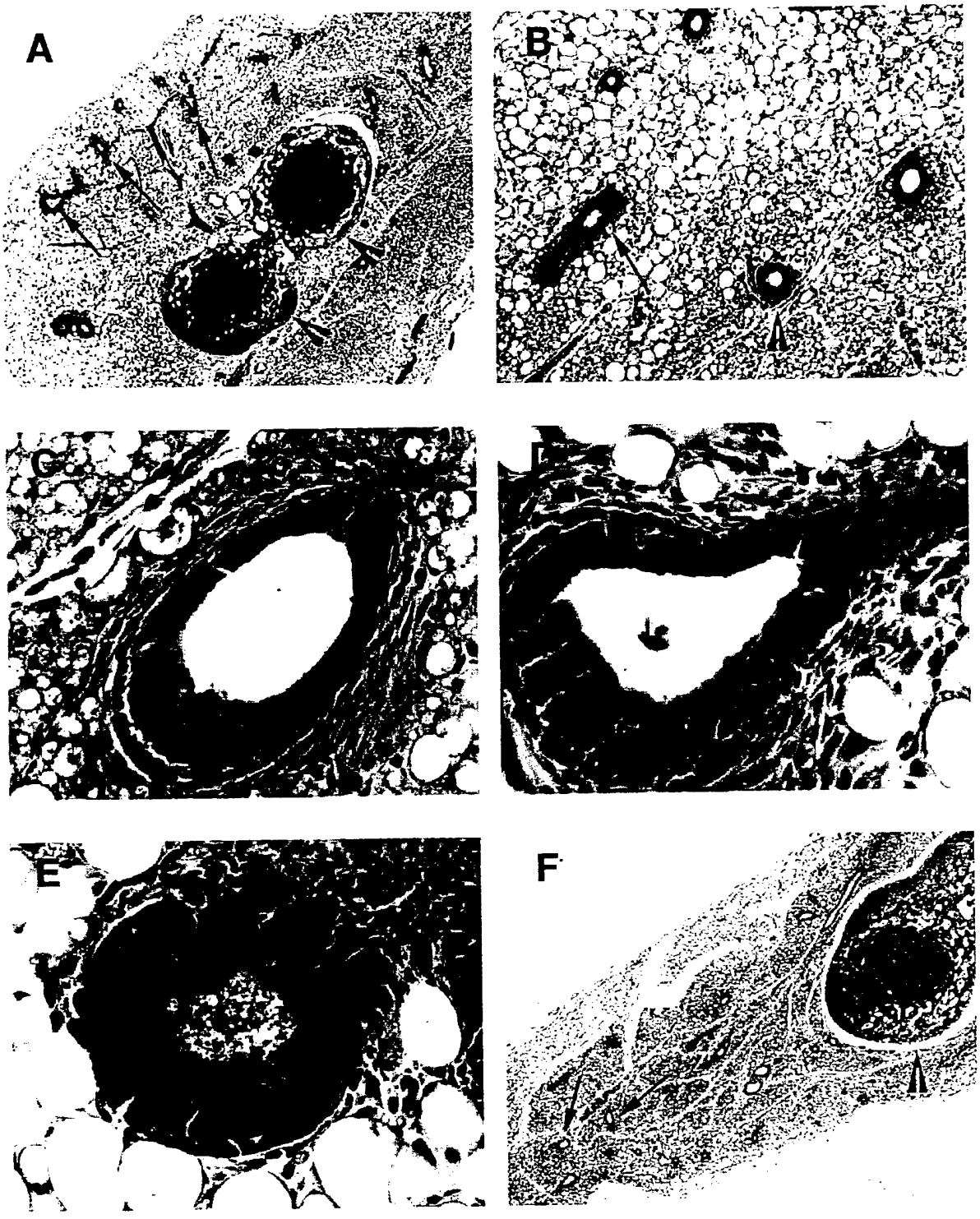
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